JOURNAL OF BACTERIOLOGY, Feb. 2002, p. 1121–1131 Vol. 184, No. 4
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.4.1121–1131.2002

Two Opines Control Conjugal Transfer of an *Agrobacterium* Plasmid by Regulating Expression of Separate Copies of the Quorum-Sensing Activator Gene *traR*

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Received 31 July 2001/Accepted 13 September 2001

Conjugal transfer of Ti plasmids from *Agrobacterium* spp. is controlled by a hierarchical regulatory system designed to sense two environmental cues. One signal, a subset of the opines produced by crown gall tumors initiated on plants by the pathogen, serves to induce production of the second, an acyl-homoserine lactone quorum-sensing signal, the quormone, produced by the bacterium itself. This second signal activates TraR, and this transcriptional activator induces expression of the *tra* regulon. Opines control transfer because the *traR* gene is a member of an operon the expression of which is regulated by the conjugal opine. Among the Ti plasmid systems studied to date, only one of the two or more opine families produced by the associated tumor induces transfer. However, two chemically dissimilar opines, nopaline and agrocinopines A and B, induce transfer of the opine catabolic plasmid pAtK84b found in the nonpathogenic *Agrobacterium radiobacter* isolate K84. In this study we showed that this plasmid contains two copies of *traR*, and each is associated with a different opine-regulated operon. One copy, $traR_{noc}$, is the last gene of the *nox* operon and was induced by nopaline but not by agrocinopines A and B. Mutating $traR_{noc}$ abolished induction of transfer by nopaline but not by the agrocinopines. A mutation in *ocd*, an upstream gene of the *nox* operon, abolished utilization of nopaline and also induction of transfer by this opine. The second copy, $traR_{acc}$, is located in an operon of four genes and was induced by agrocinopines A and B but not by nopaline. Genetic analysis indicated that this gene is required for induction of transfer by agrocinopines A and B but not by nopaline. pAtK84b with mutations in both *traR* genes was not induced for transfer by either opine. However, expression of a *traR* gene in *trans* to this plasmid resulted in opine-independent transfer. The association of $traR_{noc}$ with *nox* is unique, but the operon containing $traR_{acc}$ is related to the *arc* operons of pTiC58 and pTiChry5, two Ti plasmids inducible for transfer by agrocinopines A-B and C-D, respectively. We conclude that pAtK84b codes for two independently functioning copies of *traR*, each regulated by a different opine, thus accounting for the activation of the transfer system of this plasmid by the two opine types.

Conjugal transfer of the Ti plasmids of *Agrobacterium tumefaciens* is tightly controlled by two plasmid-encoded regulatory systems, both of which sense environmental signals. Activation of the three Ti plasmid *tra* operons, *traAFB*, *traCDG* (20), and *trb* (33), is controlled directly by quorum sensing through the acyl-homoserine lactone quormone, called *Agrobacterium* autoinducer (AAI) [*N*-(3-oxo-octanoyl)-L-homoserine lactone] (56), and TraR, a transcriptional activator of the LuxR family (22, 42). On binding AAI, TraR converts from its inactive monomeric form into its functional dimeric form (45). The activator then binds an 18-bp inverted repeat called the *tra* box located just upstream of the −35 element of its target promoters and activates transcription through interaction with RpoA (34, 45, 58; our unpublished results).

While TraR directly activates the *tra* regulon, conjugation is dependent on a second environmental signal, the conjugal opine. Opines are highly specific imine or phosphodiester conjugates of amino acids or sugars produced by crown gall tumors, neoplasias induced by *A. tumefaciens* on its host plants (reviewed in references 7 and 10). The opines are used by the bacterium as a source of carbon and energy via catabolic systems also encoded by the Ti plasmid. A subset of these compounds, the conjugal opines, also serve as signals, inducing conjugal transfer of the Ti plasmid resident in the agrobacteria that caused the tumor (reviewed in reference 18). The conjugal opines regulate transfer by controlling the expression of an operon of which *traR*, the gene coding for the quorum-sensing activator, is a member. For example, the sugar phosphodiester opines agrocinopines A and B (12), which are produced by tumors induced by *A. tumefaciens* strain C58, are used as a nutritional source by the bacterium. The *acc* operon codes for the transport and catabolism of these two opines (25, 27, 31), and expression of this set of Ti plasmid genes is controlled by the opine-responsive repressor AccR (2, 31). Agrocinopines A and B also serve as the conjugal opines of pTiC58 (16); in addition to controlling expression of the *acc* operon, AccR negatively controls expression of the adjacent, divergently oriented *arc* operon, a set of five genes of which *traR* is a member (43). Thus, in the absence of the conjugal opine, AccR represses expression of *arc*, and therefore neither *traR* nor the *tra* regulon is expressed. This arrangement of regulatory genes results in a hierarchical control process in which the opine signal is required to induce the quorum-sensing system (43).

The mechanism by which opines regulate transfer has been

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determined for three types of Ti plasmids (23, 39, 43). Although the overall strategy by which opines control quorum sensing is conserved among the three plasmids, the conjugal opines are specific to each plasmid type. Thus, agrocinopines A and B induce the transfer of the nopaline-type Ti plasmids (43), while octopine (23) and agrocinopines C and D (39) induce transfer of the octopine- and chrysopine-type Ti plasmids, respectively. Furthermore, the gene sets of which *traR* is a member differ among the Ti plasmids; while *traR* is a member of the five-gene *arc* operon on pTiC58 (43), the activator gene is a member of the 14-gene *occ* operon on the octopine-type Ti plasmids such as pTi15955, pTiR10, and pTiA6 (23). Aside from *traR*, the genes of the *occ* operon share no relatedness to those of *arc* or *acc*. Similarly, the opine-responsive transcriptional regulators are unrelated; while agrocinopines regulate gene expression via AccR, a FucR-type repressor (2), octopine serves as a coinducer for OccR, a LysR-type activator (24). This conservation of function through variable gene arrangements prompted us to propose that opine responsiveness plays an important role in the ecology of Ti plasmids by promoting transfer only in habitats where acquisition of the Ti plasmid is of benefit to the recipient bacterium (43). We also predicted that the gene arrangements resulting in such responsiveness arose by fortuitous fusions of opine-controlled gene sets to the resident allele of *traR* that controls the conjugal transfer component of the particular Ti plasmid core replicon (reviewed in reference 19).

Opine-induced conjugation is not limited to the Ti plasmids. Of particular interest is a class of *Agrobacterium* elements called the opine-catabolic plasmids, which generally are found in nontumorigenic members of the genus. These plasmids may share extensive DNA homology with Ti plasmids, but they do not confer upon their host the ability to induce tumors (6, 37). On the other hand, these extrachromosomal elements often code for the ability to utilize opines produced by tumors induced by tumorigenic agrobacteria (6, 15, 37). Some of these plasmids are transmissible by mating (11, 16), although it remains to be demonstrated that they are self-conjugal. Moreover, transfer is dependent upon opines. One such plasmid, pAtK84b, codes for the catabolism of two opine families, agrocinopines A and B and nopaline (15, 26), thus resembling nopaline-type Ti plasmids such as pTiC58. However, pAtK84b lacks the virulence regulon and T-region characteristic of Ti plasmids (6) and does not confer tumorigenicity on its host bacterium, *Agrobacterium radiobacter* strain K84. Both nopaline and agrocinopines A and B induce transfer of this plasmid from wild-type *A. radiobacter* strain K84 to an *Agrobacterium* recipient (11, 16). This observation is of interest for several reasons. First, nopaline is not known to induce the transfer of any Ti plasmid. Second, induction of conjugal transfer by more than one opine class has not been described for any Ti plasmid. Third, the region of pTiC58 responsible for catabolism of the agrocinopine opines exhibits little if any homology to pAtK84b (6, 26). Finally, two regions from pAtK84b hybridize strongly with the *traR* gene from pTiC58 (40), suggesting that transfer is directly regulated by quorum sensing and that the process is controlled by more than one copy of *traR*. We therefore sought to determine whether pAtK84b is self-conjugal, and if so, to characterize the molecular mechanism and gene arrangements responsible for the control of transfer of this plasmid by two opines of radically different chemical classes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *Agrobacterium* spp. and *Escherichia coli* as well as the plasmids used in this study are described in Table 1.

Media, substrates, and growth conditions. Cultures of *Agrobacterium* spp. and *E. coli* were grown at 28 and 37°C, respectively. L broth (low salt) (GIBCO-BRL, Rockville, Md.) was used as the rich liquid medium, and nutrient agar (Difco, Detroit, Mich.) or L agar (GIBCO) was used as the rich solid medium. AT minimal medium supplemented with 0.15% $(NH_4)_2SO_4$ and 0.2% mannitol as the sole source of carbon (ATNM) (41) was used for the growth of *Agrobacterium* strains. Medium was solidified with 15 g of agar per liter. Octopine, nopaline, and mannopine were obtained from Sigma Chemical Company, while mannopinic acid and cucumopine were the generous gifts of Yves Dessaux (ISV, CNRS, Gif sur Yvette, France). Preparations of agrocinopines A and B and of agrocinopines C and D were obtained from tumors induced on tomato plants by the nopaline-type strain C58 and the agropine-type strain Bo542, respectively (12). The agrocinopines were purified by affinity chromatography as described previously (39, 44). Purified agrocinopines from each tumor type were redissolved in 50 μl of water per g (fresh weight) of tumor tissue extracted. For *Agrobacterium*, antibiotics were used as follows (with concentrations in micrograms per milliliter): kanamycin, 100; streptomycin, 250; tetracycline, 1; carbenicillin, 100; and gentamicin, 30. For *E. coli*, antibiotics were as follows: kanamycin, 50; tetracycline, 10; and ampicillin, 100 (48). Biotin (2 μg per liter) was added to minimal medium to allow growth of *A. radiobacter* strain K84.

DNA manipulations. DNA manipulations were performed essentially as described by Sambrook et al. (46). pAtK84b was isolated and purified from NT1(pAtK84b) as described by Hayman and Farrand (26).

Southern analysis. pAtK84b and cosmid clones of this plasmid (Table 1) (6) were digested with *Hin*dIII, *Eco*RI, or *Bam*HI, as well as with combinations of these enzymes. The DNA fragments were separated by electrophoresis in 0.8% agarose gels and transferred to a charged nylon membrane (Boehringer GmbH, Mannheim, Germany). Southern blots were hybridized at medium to low stringency using two probes as described by Dessaux et al. (8). Alleles of the *traR* gene were detected using the *trlR* open reading frame (ORF) from pTi15955 (40), while *accR* was probed for using *Eco*RI fragment 26 of pTiC58, which contains the complete *accR* gene and most of *accA* (2) Probes were labeled using the digoxigenin kit from Boehringer GmbH as instructed by the manufacturer.

Bacterial matings. *Agrobacterium* donors harboring pAtK84b or its derivatives were mated with *A. tumefaciens* C58C1RS (Table 1) essentially as described by Oger et al. (40). Donor strains were incubated with shaking for 36 h in conjugal transfer induction medium, which consisted of AT medium supplemented with 0.2% glucose, 0.15% $(NH_4)_2SO_4$, and the opine being tested for induction of transfer. Octopine, nopaline, mannopine, mannopinic acid, and cucumopine were included in induction medium at a final concentration of 5 mM. Five-microliter volumes of purified solutions of agrocinopines A and B or C and D were added per milliliter of induction medium.

Nucleotide sequencing and DNA sequence analysis. Double-stranded sequencing was performed using dye terminator chemistries by the Keck Genomics Center of the University of Illinois and by Bio S&T (Lachine, Canada). DNA sequences were assembled using the Sequencher program (Gene Codes Inc., Ann Arbor, Mich.), and sequence analyses were performed using the Genetics Computer Group (Madison, Wis.) package (version 10) and DNA Strider (36). Sequences were compared with those in the databases by using the Blast protocols accessible at www.ncbi.nlm.nih.gov/BLAST (1). When necessary, local alignments were done with the ClustalW program (50).

Construction of *traR*::*lacZ* reporter fusions. The $traR_{noc}$::*lacZ* fusion was created by ligating a 1,896-bp *Bst*YI fragment containing part of the $traR_{noc}$ ORF into the compatible *Bam*HI site of pLKC482 (51) to form pPO$traR_{noc}$ (Table 1). The cloning resulted in a translational fusion between amino acid (aa) 77 of $TraR_{noc}$ and LacZ. The *ocd*::*lacZ* fusion was created by ligating a 711-bp *Eco*RI-*Msc*I fragment between the *Eco*RI and *Sma*I sites of pVIK107 (29) to form pPO*ocd* (Table 1). This cloning resulted in a translational fusion between aa 298 of ornithine cyclodeaminase (OCDase) and LacZ. The $traR_{acc}$::*lacZ* fusion was created by ligating a 2.7-kb *Hin*dIII fragment containing the first 600 bp of the activator gene into the *Hin*dIII site of pLKC482 to form pPO$traR_{acc}$ (Table 1). This cloning resulted in a translational fusion between aa 200 of $TraR_{acc}$ and LacZ. All constructs were electroporated into NT1(pAtK84b) to create single-crossover Campbell-type insertions as well as insertion disruption mutations in their respective loci (Table 1), as described previously (29, 39). The correct structures of all such insertions were confirmed by Southern analysis.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype, phenotype, or characteristic(s)[a]	Source or reference
E. coli DH5α	F^- ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44*	46
A. tumefaciens		
C58	Wild-type nopaline–agrocinopine A-B strain; Noc^+ Acc^+	Our collecton
NT1	Derivative of strain C58 cured of pTiC58	53
C58C1RS	Ti plasmid-cured C58; recipient strain for Ti plasmid matings; Rif^r Str^r	Our collection
A. radiobacter K84	Wild-type strain; produces agrocin 84; harbors three plasmids (pAtK84a [ca. 450 kb], pAtK84b [ca. 173 kb], pAgK84 [ca. 47 kb]); Noc^+ Acc^+	Our collection
Plasmids		
pUC19	ColE1-based cloning vector; Ap^r	46
pBluescript SK+	Cloning vector; Ap^r	46
pRK415	Broad-host-range cloning vector; InP1α, Tc^r	30
pKLC482	pUC8-based vector for constructing translational fusions with *lacZ*; Ap^r Km^r	51
pVIK107	R6K-based vector for constructing translational fusions with *lacZ*; Km^r	29
pMGm	Source of gentamicin resistance cassette; Ap^r Gm^r	38
pDCI41E33	Quormone reporter plasmid	28
pAtK84b	Nopaline–agrocinopine A-B catabolism plasmid; IncRh1	26
pAtK84*noc*^{c}	Nopaline-constitutive derivative of pAtK84b; Noc^c Tra^c	This study
pBC382	Cosmid clone of pAtK84b with an insert covering *acc* region; Tc^r	6
pBC420	Cosmid clone of pAtK84b with an insert covering the downstream end of the *noc* region; Tc^r	6
pPONH21b	*Hin*dIII fragment 21b from pBC382 containing the first 600 bp of $traR_{acc}$ cloned in pBluescript; Ap^r	This study
pPONH16a	*Hin*dIII fragment 16a from pBC420 containing $traR_{noc}$ cloned in pBluescript; Ap^r	This study
pPO$traR_{noc}$	*Hin*dIII fragment 16a cloned into pLKC482 to generate a $traR_{noc}$::*lacZ* fusion; Ap^r Km^r	This study
pPO$traR_{acc}$	*Hin*dIII fragment 21b cloned into pLKC482 to generate a $traR_{acc}$::*lacZ* fusion; Ap^r Km^r	This study
pPO*ocd*	711-bp *Eco*RI-*Msc*I fragment from pPONH16a containing *ocd* cloned into pVIK107 to generate an *ocd*::*lacZ* fusion; Km^r	This study
pPO$traR_{noc}$Gm	pPONH16a containing the gentamicin resistance cassette from pMGm cloned into $traR_{noc}$; Ap^r Gm^r	This study
pPON*ocd*	pAtK84b with *ocd*::*lacZ* formed by Campbell insertion of pPO*ocd, ocd*; Km^r Noc^-	This study
pPON$traR_{noc}$	pAtK84b with $traR_{noc}$::*lacZ* formed by Campbell insertion of pPO$traR_{noc}$; $traR_{noc}^-$ $traR_{acc}^+$ Noc^+ Ap^r Km^r	This study
pPON$traR_{acc}$	pAtK84b with $traR_{acc}$::*lacZ* formed by Campbell insertion of pPO$traR_{acc}$; $traR_{noc}^+$ $traR_{acc}^-$ Noc^+ Ap^r Km^r	This study
pPON$traR_{noc}$Gm	pAtK84b with $traR_{noc}$::Gm formed by marker exchange with pPO$traR_{noc}$ Gm; $traR_{noc}^-$ $traR_{acc}^+$ Gm^r	This study
pPON$traR_{noc}traR_{acc}$	pPONTraR$_{noc}$Gm with $traR_{acc}$::*lacZ* formed by Campbell insertion of pPO$traR_{acc}$; $traR_{noc}^-$ $traR_{acc}^-$ Gm^r Km^r	This study

[a] Abbreviations: acc, agrocinopine catabolism; noc, nopaline catabolism; Noc^+, utilizes nopaline; Noc^-, fails to utilize nopaline; Noc^c, constitutive for nopaline utilization; Tra^+, self-conjugal following induction with appropriate opine; Tra^-, fails to conjugate; Tra^c, constitutive for conjugal transfer.

Construction of a $traR_{noc}$ insertion mutant. A gentamicin resistance gene from pMGm (38) was inserted into the unique *Sma*I site in $traR_{noc}$ cloned in pPONH16a to form pPO$traR_{noc}$Gm (Table 1). The wild-type allele of $traR_{noc}$ on pAtK84b in strain NT1 was replaced with the $traR_{noc}$::Gm gene construct by homogenotization to create pPON$traR_{noc}$Gm, using the pPO$traR_{noc}$Gm as a suicide vector as previously described (20, 40). The correct double-crossover event was confirmed by Southern analysis.

Construction of the $traR_{noc}$ $traR_{acc}$ double mutant. Plasmid pPO$traR_{acc}$ was cointegrated into pPON$traR_{noc}$Gm to produce the double mutant pPON$traR_{noc}traR_{acc}$ (Table 1).

Selection of regulatory mutants. Regulatory mutants with mutations in the *noc* locus were obtained essentially as described by Petit and Tempé (41). Briefly, approximately 10^9 cells of NT1(pAtK84b) were spread onto plates of AB minimal medium (4) containing 0.2% arginine as the sole carbon source and incubated for up to 14 days at 28°C. Strain NT1 lacks a functional OCDase gene (9) but can utilize arginine when the nopaline-regulated OCDase coded for by the *noc* regulon of a Ti or At plasmid is expressed (14, 41). Among mutants growing on arginine plates, many are constitutive for expression of the *noc* operon because of mutations yielding nopaline-independent variants of NocR, the activator regulating expression of *noc* (35, 52). Candidate *noc*-constitutive mutants were purified on the same medium and tested for their ability to catabolize arginine. Regulatory mutations in the *arc* locus were screened for as described by Ellis et al. (13).

Assays for gene induction. Cells harboring plasmids with *traR*::*lacZ* or *ocd*::*lacZ* reporter fusions were grown to late exponential phase in ATNM medium containing the appropriate opine being tested as an inducer. The cells were collected and washed with distilled water by centrifugation and were assayed in duplicate for levels of β-galactosidase as described previously (40). Activity is expressed as units per 10^9 CFU. Each experiment was repeated a minimum of two times, and values from a representative experiment are shown.

Nucleotide sequence accession numbers. The sequences of the arc_{K84} and nox_{K84} operons, including their respective *traR* genes, have been deposited in GenBank under accession numbers AF065245 and AF065244, respectively.

RESULTS

Nopaline and agrocinopines A and B induce transfer of pAtK84b. The opine-catabolic plasmid pAtK84b is transmissible from strain K84, but only when the donor is pregrown in medium containing either nopaline or agrocinopines A and B (11, 16). However, it is not clear whether pAtK84b is self-conjugal; strain K84 harbors two other plasmids, either of which could mobilize the opine-catabolic plasmid. To address the question of self-transmissibility, we mated strain NT1

TABLE 2. Transfer of pAtK84b is induced by nopaline and agrocinopines A and B

Donor	Transfer frequency from donors grown with[a]:			
	Glucose	Nopaline	Agrocinopines A and B	Mannopine
K84	$<10^{-8}$	3×10^{-3}	2×10^{-3}	$<10^{-8}$
NT1(pAtK84b)	$<10^{-8}$	3×10^{-4}	2×10^{-3}	$<10^{-8}$
C58	$<10^{-8}$	$<10^{-8}$	2×10^{-2}	$<10^{-8}$

[a] Donors were grown in ATN medium with glucose or with glucose and the indicated opine as described in Materials and methods. Conjugation frequencies are expressed as transconjugants recovered per input donor.

(pAtK84b), which harbors pAtK84b but none of the other resident plasmids of strain K84 (26), with the recipient strain C58C1RS (Table 1). Transfer of pAtK84b was not detectable when donors were grown in and mated on medium containing only mannitol as the carbon source (Table 2). However, when pregrown in the same medium supplemented with nopaline or with agrocinopines A and B, the donor transferred pAtK84b to C58C1RS at frequencies similar to those observed in matings with strain K84 (Table 2). Thus, pAtK84b is self-conjugal, and transfer is inducible by either nopaline or the agrocinopines.

Mapping the *traR* genes on pAtK84b. By Southern analysis two fragments of pAtK84b, *Hin*dIII 16a and *Hin*dIII 21b, hybridized with a *traR* probe (40), suggesting that the plasmid codes for two copies of this regulatory gene. The fragments map to the 9 and 12 o'clock positions on the standard map of pAtK84b (6), with fragment 21b being located in the vicinity of the region believed to be responsible for transport of agrocinopines A and B and fragment 16a mapping to a region at the end of the *noc* locus, which codes for the transport and catabolism of nopaline (6) (Fig. 1).

Cloning and sequence analysis of the conjugation-regulatory loci from pAtK84b. The locations of the hybridizing segments suggest that each of the two copies of *traR* is linked to a

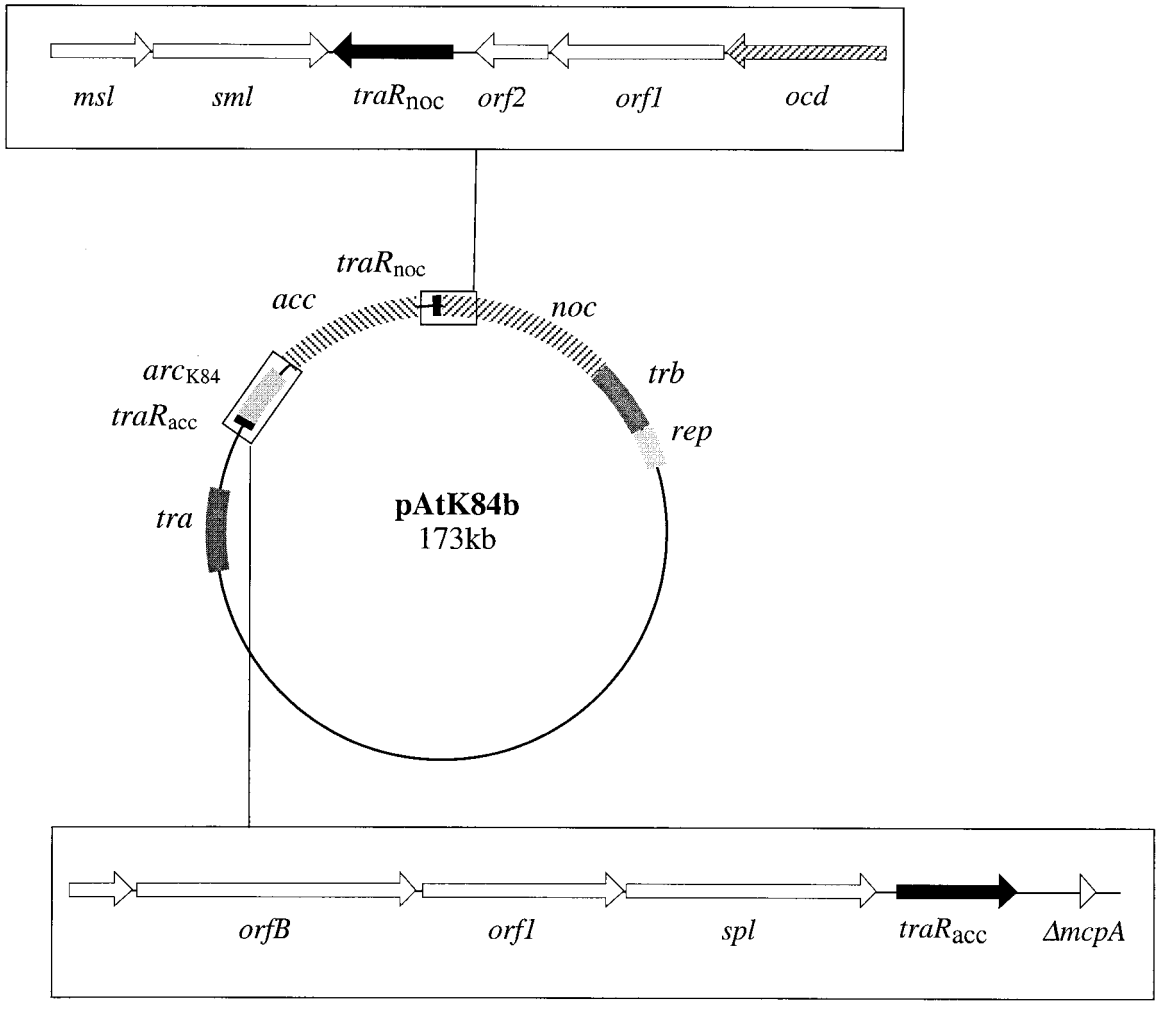

FIG. 1. Locations of the two *traR* genes on the physicogenetic map of pAtK84b. The map of pAtK84b is redrawn from reference 6. The boxed regions show the genetic organization of the two *traR* genes, indicated by the black arrows. The nature and characteristics of the additional genes, indicated by open or hatched arrows, are described in the text. Regions identified by hybridization with known loci of the nopaline-type Ti plasmid pTiC58 (6) include the following: *noc*, nopaline catabolism; *trb*, conjugal mating bridge and *traI*; *rep*, vegetative replication functions; and *tra*, conjugal DNA metabolism and *oriT*. The location of the *acc* region is inferred from genetic analyses (6, 26).

TABLE 3. Characteristics of the genes and their products located in the $traR_{acc}$ and $traR_{noc}$ regions of pAtK84b

Locus	Gene	Coordinates (bp)[a]	Translation product		Closest homologs in the data bases				
			Size (aa)	Mass (kDa)	Protein	Source	% Relatedness[b]	Accession no.	Function
$traR_{acc}$	*orfB*	292–1935	547	58.9	OrfB	pTiC58	77/85	AF010180	Unknown
	orf1	1937–3161	394	44.2	AGRL1546p	C58 linear chromosome	52/64	AE008275	Putative aldose-1-epimerase
					YihR	*E. coli* K-12	25/43	AE000463	Putative aldose-1-epimerase
	spl	3171–4637	488	53.9	SucP	pTi2608	94/95	P33910	Related to sucrose phoshorylase
					SplA	pTiC58	79/87	T03426	Related to sucrose phoshorylase
	$traR_{acc}$	4758–5465	234	26.5	$TraR_{noc}$	pAtK84b	98/98	AF065244	Quorum-sensing transcriptional activator
					$TraR_{vitis}$	pTi2608	97/97	S37463	Putative quorum-sensing transcriptional activator
					$TraR_{C58}$	pTiC58	89/92	AF010180	Quorum-sensing transcriptional activator
	mcpA[c]	5828–5927	NA[f]	NA	McpA	pTiC58	87/87	AF010180	Methyl-accepting chemotaxis protein
$traR_{noc}$	*ocd*[d]	1–925	>308	>33.6	OCDase	pTiC58	97/98	P09773	Ornithine cyclodeaminase
	orf1	945–2075	376	41.5	None	NA	NA	NA	Hypothetical protein
	orf2	2005–2403	133	14.6	None	NA	NA	NA	Hypothetical protein
	$traR_{noc}$	2542–3246	234	26.5	$TraR_{acc}$	pAtK84b	98/98	AF065245	Quorum-sensing transcriptional activator
					$TraR_{vitis}$	pTi2608	96/97	S37463	Quorum-sensing transcriptional activator
					$TraR_{C58}$	pTiC58	89/82	AF010180	Quorum-sensing transcriptional activator
	sml	4303–3281	340	31.2	SmoC	*Xanthomonas* spp.	30/48	AF018073	Repressor
	msl[e]	4860–4308	>183	>19.6	Mas1′	pRi8196	32/50	P50201	Opine oxidoreductase

[a] Numbers represent the first nucleotide of the translational start codon and the last nucleotide of the last coding triplet unless otherwise noted.
[b] Numbers represent percent amino acid identities/percent amino acid similarities, including conserved substitutions.
[c] A 90-bp segment of nucleotide sequence.
[d] Sequence represents the last 925 bp of the *ocd* gene.
[e] Sequence represents the first 579 bp of the *msl* gene.
[f] NA, not applicable.

locus required for catabolism of one of the two conjugal opines. To assess this hypothesis, we sequenced the two *traR* alleles and the regions surrounding these genes.

Analysis of the sequence of a 6,080-bp segment including fragment 21b identified four complete ORFs, all oriented in the same direction (Fig. 1). The last ORF, which we named $traR_{acc}$, could encode a 234-residue protein closely related to the products of other *traR* genes from *Agrobacterium* Ti plasmids (Table 3). $traR_{acc}$ is preceded by a 1,464 bp-ORF, *spl* (Fig. 1), that could encode a 488-residue protein that is 87% similar in sequence to the product of *splA* from the *arc* operon of pTiC58 (Table 3 and Fig. 2). *spl* is preceded by an 1,182-bp ORF, *orf1* (Fig. 1), which is not present in the *arc* operons of pTiC58 and pTiChry5 (Fig. 2 and data not shown). The 394-residue translation product of this ORF is 52% identical and 64% similar in sequence to the product of an ORF, AGR-L-1564, located on the linear chromosome of strain C58 (Table 3). The products of both ORFs also are related to YihR, a gene product of *E. coli* K-12 that is itself related to aldose-epimerases (Table 3). *orf1* is preceded by a 1,641-bp ORF, *orfB* (Fig. 1), that could code for a protein of 547 residues, the sequence of which is 85% similar to the product of *orfB* from the *arc* operon of pTiC58 (Table 3 and Fig. 2). Directly downstream of $traR_{acc}$ is a 90-bp nucleotide sequence that is identical to a portion of *mcpA*, a gene located immediately downstream of $traR_{C58}$ in the *arc* operon of pTiC58 (Fig. 2). Finally, the 3′ end

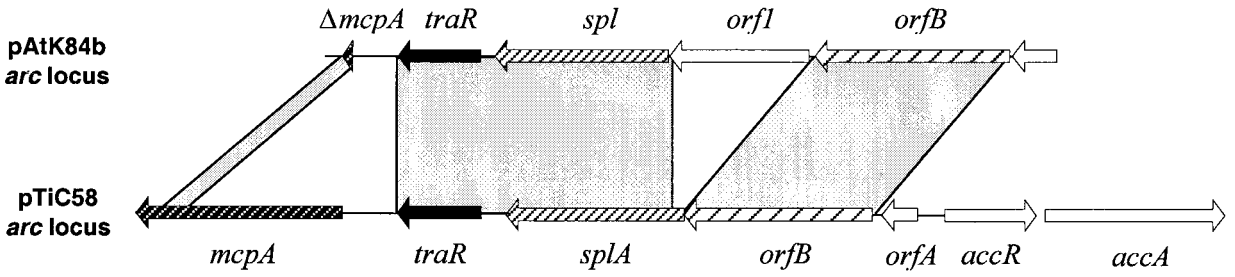

FIG. 2. Organizational similarities between the *arc* operons of pAtK84b and pTiC58. The structure of the *arc* operon of pTiC58 is redrawn from Piper et al. (43). Genes the products of which are related at the amino acid sequence level are depicted by arrows with the same fill. Open arrows indicate genes that are not conserved between the two *arc* operons. Regions of conserved nucleotide sequence between the two *arc* operons are shown by shaded boxes connecting the two sets of genes.

of an incomplete ORF oriented in the same transcriptional direction is located just upstream of *orfB* (Fig. 1). The deduced amino acid sequence of this partial ORF is not related to any protein in the databases. Based on sequence and organizational similarities with the *arc* operon of pTiC58 (Fig. 2), we named this gene set of pAtK84b arc_{K84}.

Nucleotide sequence analysis of a 4,860-bp region including *Hin*dIII fragment 16a identified four complete and two incomplete ORFs (Fig. 1). One of the incomplete ORFs, located on the far right-hand (clockwise) side could code for a protein that is closely related to the product of the *ocd* gene, which is the last ORF in the *nox* operon of pTiC58 (Table 3). The four genes of this operon from pTiC58 code for the enzymes responsible for degrading nopaline to proline (47, 55), with *ocd* specifying OCDase (47), an enzyme required for the cyclization of ornithine to proline (55). The middle ORF, which we named $traR_{noc}$, is transcribed in the same direction as *ocd* (Fig. 1). Like for $traR_{acc}$, the 234-residue translation product of this gene is closely related to the products of other *traR* genes (Table 3). $traR_{noc}$ and *ocd* are separated by two slightly overlapping ORFs, *orf1* and *orf2*, of 1,128 and 399 bp, respectively. The 376- and 133-residue translation products of these two ORFs are not related to any sequences in the protein databases. $traR_{noc}$, *orf1*, *orf2*, and *ocd* are oriented in the same direction and could be transcribed as an operon (Fig. 1). The remaining two ORFs, *sml* and *msl*, located on the left-hand side of the sequenced region, are oriented convergently to the first four ORFs (Fig. 1). *sml* (for *smoC*-like) could encode a 340-residue protein weakly related to SmoC, a sorbitol-responsive repressor from *Xanthomonas* species, while *msl* (for mannopine synthase-like) could code for a protein related to the N-terminal portion of Mas1′, an oxidoreductase required for the synthesis of mannopine, another opine coded for by the T regions of several Ti and Ri plasmids (Table 3). A segment of the product of the *msl* ORF also is related to a domain strongly conserved among the phosphogluconate mutase family.

The conjugal opines induce expression of their cognate *traR* genes. In the three examples described to date, opines regulate conjugation of Ti plasmids by controlling expression of *traR* (23, 39, 43). Given that pAtK84b contains two full-sized copies of the *traR* gene and is induced for transfer by two opines we reasoned that expression of each gene would respond to one or the other of the conjugal signals.

$traR_{noc}$ is located directly downstream from an *ocd* homolog within the nopaline-catabolic region of pAtK84b, suggesting that the expression of this allele is controlled by nopaline. We tested this hypothesis by determining the influence of the arginyl opine on the expression of the *ocd* and $traR_{noc}$ genes of pAtK84b. As assessed by analysis of β-galactosidase activity from *lacZ* reporter fusions integrated into pAtK84b, both genes are expressed at very low basal levels in cells grown with mannitol as the sole carbon source (Table 4). However, addition of nopaline to the growth medium resulted in 15- to 20-fold increases in the levels of expression of these two genes. No other opine tested, including agrocinopines A and B, induced the expression of either gene (Table 4).

On pTiC58 the single copy of *traR* is located within *arc*, an operon of five genes the expression of which is induced by agrocinopines A and B (43). Given the relatedness between arc_{C58} and arc_{K84} (Fig. 2), we reasoned that expression of $traR_{acc}$ is controlled by the agrocinopines in a similar manner. As assessed by analysis of β-galactosidase activity, $traR_{acc}$ was expressed at very low levels in the absence of the agrocinopines. However, addition of these sugar phosphodiester

TABLE 4. Nopaline and agrocinopines A and B induce expression of only their cognate *traR* genes on pAtK84b

Plasmid	*lacZ* fusion to:	β-Galactosidase activity following growth with[a]:				
		Glucose	Nopaline	Agrocinopines A and B	Agrocinopines C and D	Mannopine
pAtK84b	None	<1	<1	<1	NT[b]	<1
pPON*ocd*	*ocd*	<1	22	<1	NT	<1
pPON$traR_{noc}$	$traR_{noc}$	<1	15	<1	NT	<1
pPON$traR_{acc}$	$traR_{acc}$	<1	<1	8	<1	<1
pPON$traR_{noc}traR_{acc}$	$traR_{acc}$	<1	<1	8	NT	NT

[a] Cells were grown in ATN minimal medium supplemented with glucose or glucose and the indicated opine. β-Galactosidase activities, determined as described in Materials and Methods, are expressed as units per 10^9 CFU.
[b] NT, not tested.

TABLE 5. Growth of strains harboring pAtK84b and its *noc* and $traR_{noc}$ mutants on medium containing arginine and nopaline

Plasmid[a]	Growth on ATN minimal medium containing as sole carbon source[b]:				
	Glucose	Arginine	Nopaline	0.05× nopaline[c]	0.05× nopaline + arginine
pAtK84b	++++	−	+++	+/−	+++
pPON*ocd*	++++	−	−	NT[d]	NT
pPON$traR_{noc}$	++++	−	+++	NT	NT
pAtK84b*noc*[c]	++++	++	+++	+/−	++

[a] Phenotypes were tested in the *A. tumefaciens* NT1 chromosomal background.
[b] Growth, assessed on ATN agar medium containing glucose at 0.2% or opines or arginine at 1 mg per ml, is expressed as follows: ++++, very good; +++, good; ++, easily detectable; +/−, barely detectable.
[c] Nopaline at a 0.05-mg/ml final concentration.
[d] NT, not tested.

opines to the growth medium resulted in an eightfold increase in the expression level of the *lacZ* fusion (Table 4). No other opines, including nopaline, mannopine, and the closely related sugar phosphodiester opines agrocinopines C and D, induced expression of the $traR_{acc}$::*lacZ* reporter (Table 4 and data not shown).

Mutations in the *traR* genes abolish induction of transfer but only by the cognate controlling opine. In addition to creating reporters, the *ocd*::*lacZ* and $traR_{noc}$::*lacZ* fusions produced *ocd*- and $traR_{noc}$-null mutants of pAtK84b. Strains harboring the *ocd* mutant plasmid failed to catabolize nopaline, while the strain harboring the $traR_{noc}$ mutation grew as well as the wild type with the opine as the sole carbon source (Table 5). Both mutant plasmids failed to transfer from donors pregrown in medium containing nopaline (Table 6). Providing a constitutively expressing copy of *traR* in *trans* restored conjugal transfer to both mutant plasmids (data not shown). Moreover, the complemented mutant plasmids transferred even in the absence of induction by nopaline.

In a manner analogous to that for the $traR_{noc}$ fusion, the $traR_{acc}$::*lacZ* construct created a $traR_{acc}$-null mutation. This mutant plasmid failed to transfer from donors pregrown in medium containing agrocinopines A and B (Table 6). However, providing $traR_{acc}$ or $traR_{noc}$ in *trans* to the mutant plasmid restored conjugal transfer (data not shown). Moreover, as with the $traR_{noc}$ mutant, transfer of the complemented $traR_{acc}$::*lacZ* construct is independent of induction by agrocinopines A and B.

***$traR_{noc}$* and *$traR_{acc}$* are regulated independently.** The presence of two copies of *traR*, coupled with our observation that these two genes code for functional proteins, suggested that the two alleles on pAtK84b are regulated independently by the two conjugal opines. We tested this hypothesis by assessing the influence of the alternate conjugal opine on the transfer of the *traR*-null mutant plasmids. The mutant plasmids transferred at frequencies similar to those of pAtK84b when the donors were pregrown in medium containing the alternate conjugal opine, the cognate opine for the wild-type copy of *traR* (Table 6). Thus, pPON$traR_{acc}$ (pAtK84b$traR_{acc}$::*lacZ*) transferred from donors induced with nopaline but not with agrocinopines, while pPON$traR_{noc}$ (pAtK84b$traR_{noc}$::*lacZ*) transferred from donors induced with agrocinopines A and B but not with nopaline. Growth in medium containing both opines did not increase the frequency of transfer of pAtK84b from strains K84 or NT1(pAtK84b) (data not shown).

While derivatives of pAtK84b disrupted in one of the two *traR* genes transferred when induced with the second opine, pPON$traR_{noc}traR_{acc}$ (pAtK84b$traR_{acc}$::*lacZ-traR*$_{noc}$::Gm), in which both *traR* genes are mutant (Table 1), failed to transfer from donors grown in medium containing either opine alone or in medium containing both opines (Table 6). However, transfer of this mutant plasmid was restored by providing either *traR* gene in *trans* (data not shown).

Isolation of opine-regulatory mutants constitutive for conjugal transfer. Agrocinopines A and B control transfer of pTiC58 by regulating expression of the *arc* operon, and therefore *traR*, via the opine-responsive repressor AccR (43). Transfer-constitutive (Tra^c) mutants of pTiC58 are easily isolated by selecting for rare transconjugants following matings conducted in the absence of the inducer opines (13). Such mutants invariably contain null alleles of *accR* (reference 2 and unpublished results). However, despite repeated attempts, we were unable to isolate such spontaneous Tra^c mutants of pAtK84b.

Expression of the *noc* and *nox* operons of pTiC58 is controlled by NocR, a LysR-type transcriptional activator (35, 52). Mutants of pTiC58 exhibiting constitutive expression of the two operons can be obtained by selecting for growth on medium containing arginine as the sole carbon source (14, 41). Among such variants are those that presumably code for a constitutive form of NocR (NocR^c) that no longer requires the opine ligand to activate expression of the *noc* and *nox* operons. Such Noc^c mutants also utilize octopine, a chemically related but noninducing arginyl opine (41). Given that nopaline regulates the expression of *ocd* and $traR_{noc}$ on pAtK84b, we predicted that this plasmid codes for a NocR-like activator and that at least some Noc^c mutants of this plasmid should be constitutive for conjugal transfer.

NT1(pAtK84b) did not grow on minimal medium containing

TABLE 6. Opine induction of conjugal transfer is mediate only through the cognate *traR* gene of pAtK84b

Plasmid	Mutant *traR*	Frequency of transfer following growth with[a]:			
		Glucose	Nopaline	Agrocinopines A and B	Mannopine
pAtK84b	None	$<10^{-8}$	2×10^{-3}	3×10^{-3}	$<10^{-8}$
pPON*ocd*	None[b]	$<10^{-8}$	$<10^{-8}$	2×10^{-3}	$<10^{-8}$
pPON$traR_{noc}$	$traR_{noc}$	$<10^{-8}$	$<10^{-8}$	3×10^{-3}	$<10^{-8}$
pPON$traR_{acc}$	$traR_{acc}$	$<10^{-8}$	3×10^{-3}	$<10^{-8}$	$<10^{-8}$
pPON$traR_{noc}traR_{acc}$	$traR_{noc}$, $traR_{acc}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$

[a] Donors were grown in ATN with glucose or glucose and the indicated opine. Conjugation frequencies are expressed as transconjugants recovered per input donor.
[b] The mutation in *ocd* is polar on $traR_{noc}$; see text for details.

TABLE 7. Mutants of pAtK84b constitutive for nopaline catabolism are constitutive for conjugal transfer

Plasmid	Transfer frequency following growth with[a]:		
	Glucose	Nopaline	Agrocinopines A and B
pAtK84b	$<10^{-8}$	4×10^{-3}	3×10^{-3}
pAtK84b*noc*^{c}	6×10^{-5}	2×10^{-3}	3×10^{-3}

[a] Donors were grown in ATN minimal medium containing glucose or glucose and the indicated opine. Conjugation frequencies are expressed as transconjugants recovered per input donor.

arginine or octopine as the sole carbon source (Table 5). However, after incubation for 1 to 2 weeks, about 100 colonies appeared on arginine plates inoculated with ca. 10^{10} cells of NT1(pAtK84b). Ten such colonies from each of four selection plates were isolated and purified. All 40 clones utilized arginine and octopine in the absence of nopaline (Table 5 and data not shown). Of the Noc^c mutants tested, all transferred pAtK84b even when pregrown in the absence of nopaline (Table 7 and data not shown). Moreover, while NT1(pAtK84b) produces barely detectable amounts of AAI when grown without a conjugal opine (3), the Noc^c mutants produced large amounts of the quormone under these noninducing conditions (data not shown). However, transfer frequencies, as well as levels of AAI produced by these Noc^c Tra^c mutants under noninducing conditions, were consistently lower than those of NT1(pAtK84b) grown with nopaline (Table 7 and data not shown). Transfer frequencies and signal production exhibited by these Noc^c Tra^c mutants could be fully induced by providing nopaline or agrocinopines A and B in the growth medium (Table 7).

DISCUSSION

In this study we show that regulation of transfer of pAtK84b by two opines, first reported by Ellis et al. (16), results from the independent control of two copies of *traR*, each by one of the two tumor-produced opine signals. In this regard, pAtK84b is the first of the Ti-like plasmids from the genus *Agrobacterium* to be shown to contain two functional copies of the quorum-sensing activator. The octopine-type Ti plasmids pTi15955 (40) and pTiR10 (57) also contain two independently regulated *traR* genes. However one such gene, called *trlR*, codes for a mutant protein that lacks the C-terminal DNA binding domain and consequently fails to activate transcription (40, 57). The product of this gene is induced by mannopine, a member of the mannityl opine family, and strongly inhibits activity of the functional quorum-sensing activator coded for by the *traR* gene located within the octopine operon. Although it is the first example, it is likely that pAtK84b is not unique; pAtK112, an opine catabolic plasmid in a second isolate of *A. radiobacter*, is induced for transfer by both nopaline and agrocinopines A and B (16), suggesting that this element also contains two functional copies of *traR*. Moreover, *A. radiobacter* K299, which catabolizes both nopaline and agrocinopines A and B, contains at least two copies of the *traR* gene (40). Like K84, both K112 and K299 were isolated from orchards in South Australia. However, it is unlikely that the plasmid in either isolate is identical to pAtK84b; both pAtK112 and pAtK299 confer susceptibility to agrocin 84 (26; J. G. Ellis, personal communication).

Among the Ti plasmids examined to date, opines control conjugation because *traR* is located within an operon regulated in response to the tumor signal (Fig. 3) (23, 39, 43). Such also is the case for pAtK84b; clearly, expression of the two *traR* genes is induced by one or the other of the two conjugal opines (Table 4), and each is a member of a gene set regulated by one or the other opine inducer (Fig. 1). The agrocinopine-responsive copy, $traR_{acc}$, is located within a group of four genes all oriented in the same direction. Within this group, the two-gene set of $traR_{acc}$ and *spl* is found in the *arc* operons of pTiC58 and pTiChry5 (Fig. 2 and 3) (39, 43). Moreover, arc_{K84} contains a homolog of *orfB* and remnants of *mcpA*, the terminal gene of the *arc* operon of pTiC58 (Fig. 3). In pTiC58 (43), and probably also in an otherwise unrelated Ti plasmid, pTiChry5 (39), the *arc* operon, and therefore *traR*, is negatively regulated by AccR, the transcriptional repressor for which agrocinopines serve as the inducers (2). Thus, in the presence of the sugar phosphodiester opines, AccR releases from the *arc* promoter, the operon is expressed, and TraR is produced at levels sufficient to activate expression of the *tra* regulon. On pTiC58, *accR*, the gene coding for the opine-responsive repressor, is the first member of the *acc* operon located just upstream of and in the opposite orientation to *arc* (31). The *acc* operon of this Ti plasmid, in turn, is responsible for the uptake and catabolism of agrocinopines A and B (25, 27, 31). Thus, *arc* and *acc* with its attendant *accR* form a tightly linked but separately transcribed pair of operons on pTiC58 that couple opine catabolism to conjugal transfer. While $traR_{acc}$ and portions of *arc* are conserved on pAtK84b, it is not at all clear how the agrocinopines regulate expression of this operon on the opine-catabolic plasmid. pAtK84b, although conferring uptake of agrocinopines A and B (26), does not hybridize significantly with elements of the *acc* operon from pTiC58 (6, 26). Consistent with this observation, acc_{K84}, the to-date-uncharacterized agrocinopine transport locus of pAtK84b, does not confer uptake of or sensitivity to the toxic opine analog agrocin 84 (26). Susceptibility to this antibiotic is a hallmark associated with all other known agrocinopine catabolism systems, including those of pTiC58, pTiBo542, and pTiChry5 (21, 26, 39). Furthermore, the region immediately upstream of the *arc* operon on pAtK84b does not contain a homolog of *accR* (data not shown). By Southern analysis we did detect two regions of pAtK84b that hybridized weakly with an *accR* probe (data not shown). However, neither region is linked closely to arc_{K84}, suggesting that if either segment does encode the agrocinopine-responsive regulatory gene, it, and perhaps the rest of acc_{K84} has diverged from the *acc* operon of pTiC58 and, unlike the Ti plasmid, is spatially separated from *arc*. Finally, we were unable to isolate agrocinopine-independent transfer-constitutive mutants of pAtK84b. Such mutants, invariably with lesions in *accR*, are obtained readily from pTiC58 (13; our unpublished results). Despite these differences, the structural similarities between arc_{K84}, arc_{C58}, and arc_{Chry5} (Fig. 3) clearly indicate that the three gene sets have evolved from a common ancestral operon.

Expression of the second activator gene, $traR_{noc}$, is induced by nopaline but not by agrocinopines A and B (Table 3). The gene is located downstream from and in the same transcrip-

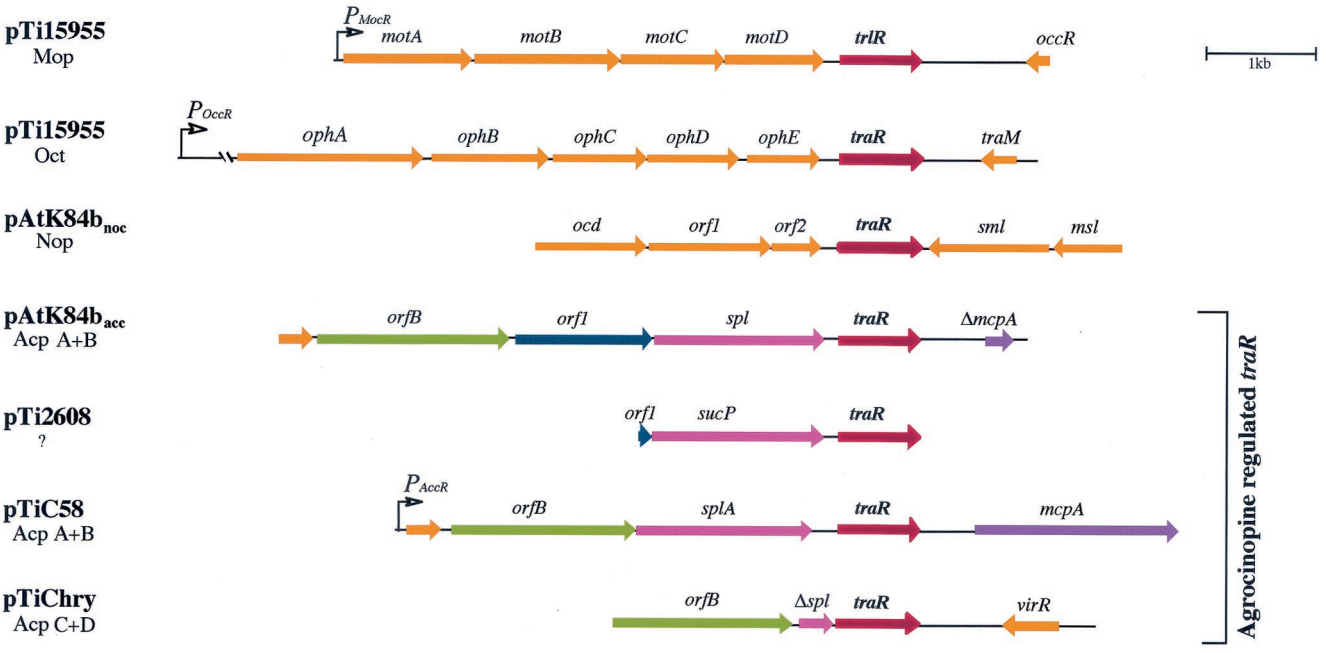

FIG. 3. Gene compositions and relationships among the conjugal control regions from Ti and At plasmids found in members of the genus *Agrobacterium*. Each gene set represents an operon that contains a copy of *traR*, as indicated by the red arrow. Yellow arrows represent genes that are unique to any one of the conjugal control operons. Arrows in green, blue, purple, and pink represent genes exhibiting various levels of conservation among the three iterations of the *arc* operon. Black open arrows labeled P represent promoters defined by transcript or genetic analyses (23, 40, 43). The mannopine (Mop)-associated *trlR* region of pTi15955 is from reference 40, the octopine (Oct)-associated *traR* region of pTi15955 is from reference 23, the nopaline (Nop)- and agrocinopine (Acp) A- and B-associated *traR* regions of pAtK84b are from this work, the *traR* region of *A. vitis* is from reference 21, the Acp A- and B-associated *traR* region of pTiC58 is from reference 43, and the Acp C- and D-associated *traR* region of pTiChry5 is from reference 39.

tional orientation as *ocd*, the gene coding for OCDase, an enzyme essential for catabolism of nopaline (47). On pTiC58, the genes required for nopaline catabolism are organized as two operons separated by about 5 kb of sequence of unrelated function. The first operon, *noc*, codes for the four genes of the transport system (54), while the second, *nox*, codes for the three enzymes, including OCDase, required for catabolism of the opine (55). Hybridization analysis suggests that the nopaline catabolism region of pAtK84b is organized in a similar manner (6). Two lines of evidence indicate that $traR_{noc}$ is part of the *nox* operon. First, an insertion mutation in ocd_{K84} abolishes nopaline-inducible transfer but has no effect on transfer induced by agrocinopines A and B (Table 6). We take this to mean that the mutation in *ocd* is strongly polar on nopaline-induced expression of the downstream $traR_{noc}$ gene. Consistent with this conclusion, the *ocd* mutant can be complemented for transfer by $traR_{noc}$ expressed in *trans*. That the *lacZ* fusion to *ocd* is induced by nopaline (Table 4) and that the mutation arising from this fusion abolishes nopaline utilization (Table 5) confirm that this gene is indeed part of the *noc* system. Second, mutants of pAtK84b constitutive for nopaline catabolism also transfer in the absence of induction by either conjugal opine (Table 7), However, as first noted by Ellis et al. (15), such Noc^c variants are not fully constitutive for transfer; growing donors harboring such mutant At plasmids with nopaline increases the transfer frequency by almost two orders of magnitude (Table 7). We suspect that the *noc* regulon of pAtK84b is controlled by a transcription factor similar, if not identical, to NocR, the LysR-like activator responsible for controlling *noc* of pTiC58 (35, 52). NocR is closely related to OccR, the LysR-like activator of the octopine operons of Ti plasmids such as pTiR10 and pTi15955 (24, 52). A subset of such octopine-independent mutants of OccR are only partially constitutive and respond positively to addition of octopine (5).

While pAtK84b contains two copies of *traR*, the plasmid contains only one copy of the genes comprising the conjugation system. Southern analyses indicate that the *traAFB* and *traCDG* operons are arranged in a manner similar to that in pTiC58 and are located in the region just anticlockwise from $traR_{acc}$ (Fig. 1) (6). The *trb* operon, which also codes for TraI (28), the acyl-homoserine lactone quormone synthase, is located at about two o'clock on the map of pAtK84b (Fig. 1) (6) and is linked closely to the replication region, a feature that is conserved in all Ti plasmids analyzed to date (32). Thus, it is likely that the two TraR paralogs, the expression of each of which is induced by its own opine, activate the single *tra* regulon.

In the four Ti plasmids for which nucleotide sequences are available, *traR* always is oriented opposite to and located within a few kilobases downstream of the *traAFB* operon 43, 49, 59; our unpublished results). On pAtK84b, $traR_{acc}$ associated with the arc_{K84} operon represents this classical arrangement (Fig. 1), and thus the system of this plasmid derives from a lineage that includes the conjugal control regions of pTiC58 (43), pTiSAKURA (49), pTiChry5 (39), and pTiBo542 (our unpublished results). Significantly, conjugal transfer of each of

these plasmids is induced by one or the other of the two families of agrocinopine-type opines (16, 39). Interestingly, the arc_{K84} operon most closely resembles the *sucP-traR* gene set found on several Ti plasmids from *Agrobacterium vitis* (Fig. 3) (21). Moreover, $TraR_{acc}$ from pAtK84b is most closely related to the TraR-like gene product of these Ti plasmids (Table 3). However, the TraR products from the *A. vitis* Ti plasmids apparently are not functional (43), and the *sucP-traR* gene set on these elements may be vestigial.

While arc_{K84} represents another iteration of the *arc*-type conjugal control region, the association of $traR_{noc}$ with the nopaline catabolism system of pAtK84b is novel in its arrangement. Nopaline is not known to induce transfer of any Ti plasmid, although this opine does activate conjugation of the opine-catabolic plasmid pAtK112 (16). The association of $traR_{noc}$ with the nopaline catabolism regulon is conceptually reminiscent of the association of $traR_{occ}$ with the octopine system of octopine-type Ti plasmids (23). However, although the organization of the downstream elements of *occ* resembles that of the downstream elements of *nox*, it is unlikely that the associations of $traR_{noc}$ and of $traR_{occ}$ with their respective *noc* and *occ* systems derive from a common lineage. While $traR_{noc}$ is separated from ocd_{noc} by two ORFs, $traR_{occ}$ is located almost 8 kb downstream of ocd_{occ}, with the intervening region coding for six genes on pTiR10 (Fig. 3) (23). Moreover, the two ORFs between ocd_{noc} and $traR_{noc}$ on pAtK84b are not related to any of the six genes located between ocd_{occ} and $traR_{occ}$ (Fig. 3). Thus, the association of *traR* with the *noc* and *occ* systems most likely arose following the divergence of these two opine catabolism systems from some common ancestor. The *nox-traR*$_{noc}$ gene set also is novel with respect to placement; this *traR* control operon is not closely linked to the *traAFB* operon (Fig. 1). Instead, $traR_{noc}$ abuts two ORFs in the opposite orientation located at the edge of a region of pAtK84b showing no detectable relatedness with pTiC58 or the octopine-type Ti plasmid pTiAch5 (6).

Clare et al. (6) suggested that pAtK84b arose as a deletion derivative of a nopaline-type plasmid such as pTiC58. We think that such an origin is unlikely for several reasons. First, while the *tra* regulon, replication region, and nopaline catabolism system of pAtK84b are closely related to those of pTiC58, the agrocinopine utilization system differs significantly in function and sequence from that of the nopaline-type Ti plasmids (6, 26). Second, although the *arc* conjugal control regions of pTiC58 and pAtK84b clearly share a common phylogeny (Fig. 3), they have diverged significantly. Third, pTiC58 lacks any association of *traR* with the nopaline catabolism system, suggesting that the *nox-traR* arrangement on pAtK84b is novel and arose independently. Finally the *noc-nox-traR* interval of pAtK84b is contiguous to a ca. 10-kb region of the plasmid sharing no detectable relatedness to the nopaline-type Ti plasmids (6). On pTiC58 *nox* is contiguous with the T region, a segment crucial for pathogenicity that is not present on pAtK84b. This last observation suggests that this portion of pAtK84b could have derived from some other type of nopaline-catabolic element of *Agrobacterium*. We think it likely that pAtK84b is chimeric and, as such, has arisen as an assemblage of segments of other Ti and At plasmids.

pAtK84b is central to the biology of strain K84. This bacterium, although itself unable to induce tumors, can, by virtue of its ability to catabolize nopaline and agrocinopines A and B, co-opt those galls produced by nopaline-type pathogens. Moreover, agrocin 84 produced by strain K84 is lethal to and targets specifically pathogenic nopaline-agrocinopine-type strains of *A. tumefaciens* (17). The two traits combined allow strain K84 to occupy the niche provided by the tumor and to eliminate competition by the pathogenic agrobacteria that induced the neoplasia (18). In this regard, the bacterium by virtue of its resident plasmids is a highly evolved fratricidal parasite. Finally, pAtK84b, the plasmid responsible for these catabolic traits, utilizes both opines as signals to induce conjugal transfer and can be induced for transfer within nopaline-agrocinopine type tumors or within tumors induced by other agrobacteria that produce only one or the other of the two opine types.

ACKNOWLEDGMENTS

This work was supported by grant R01 GM52465 from the NIH and by Hatch project no. 15-340CS to S.K.F. P.O. was supported in part by grant 99I-59-4 CS from C-FAR, State of Illinois, to S.K.F.

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